Electron microscopy of host tissue deterioration in Dutch elm disease

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IN DUTCH ELM DISEASE.

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Electron microscopy of host tissue deterioration in Dutch elm disease

by

Marion Edward Jones, Jr.

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

For a half-century, Dutch elm disease, caused by Ceratocystis ulmi (Buism.) C. Moreau, has been researched. Numerous descriptions of C. ulmi-infected elm wood have been made at the light microscope level. Most of these studies have dealt with infected tissue at advanced stages of disease. Recently, observations at the electron microscope level were made with emphasis placed on the first two weeks after infection, or the period before external symptoms were expressed (MacDonald, 1970). An attempt was made also to determine tissue reaction differences between susceptible and resistant hosts. This first fine-structural study established that during the first two weeks after infection several phenomena were expressed by host tissue and by the disease organism: formation of tyloses, accumulation of apparent phenol-like substances in the vacuoles of parenchyma cells, darkening of the vessel pit "membranes", and the penetration of pit "membranes" and cell walls by the fungus. With the present study, I have added the resolution of the electron microscope to clarify and add to previous descriptions of diseased host tissue after the onset of external symptoms and to determine differences, if any, between susceptible and resistant hosts.

I correlated my research closely with the previous electron microscope study of early diseased elm tissue...
(MacDonald, 1970). Thus the two studies compliment each other; mine beginning at the point where the previous study ended. Therefore, I felt it important to focus upon certain questions raised by MacDonald's previous investigation: location of discoloration within the cell, sequence of tissue deterioration, apparent lack of gum-like material in diseased vessels, and differences in responses between susceptible and resistant hosts. Thus, I formulated the following objectives for this study:

1. to follow the events that sequentially take place during the diseased-induced death of parenchyma cells;
2. to determine in which cellular locations and structures tissue discoloration takes place;
3. to note the type of abnormal material, if any, accumulating in diseased vessels;
4. to note differences, if any, between host reactions of the susceptible Ulmus americana L. and the resistant U. carpinifolia Gleb. 'Christine Buisman'.
REVIEW OF LITERATURE

General Aspects of Host Tissue Reaction

Much literature is available on host tissue reactions in vascular wilt diseases (Dimond, 1955; Beckman, 1964; Dimond, 1967; MacDonald, 1970). However, I will confine this review to the issues directly related to my study objectives.

Deterioration of diseased tissue

During the course of vascular wilt diseases, host xylem tissue changed from an active, dynamic, functional area to one of nonfunctional, dead and deteriorating cells. Deterioration was not limited to the once living parenchyma cells. Cell wall layers in oak were degraded, apparently, by fungal enzymes produced by the oak wilt fungus (Sachs et al., 1970). Polarized light microscopy indicated that cellulose had been degraded.

The ability of different pathogens to attack host cell walls varies. Some supplemented their otherwise restricted diet by utilizing products of hydrolysis of cell wall polysaccharides (Husain and Dimond, 1958a, b); however, others degrade cell walls but did not utilize the hydrolytic products as nutrients (Husain and Kelman, 1958; Kelman and Cowling, 1965). With certain wood decay fungi, the cellulose was degraded as elongated cavities were formed in the secondary walls. Cellulose of the secondary wall
was dissolved enzymatically to form cavities that were oriented either helically or parallel to the long axis of the cell. Such attacks suggested hydrolysis along planes determined by the structural orientation of the cellulose (Coté, 1968). According to Gagnon (1967a), pectin, cellulose, and lignin in diseased elm were degraded as the cell walls were attacked. Reports by Ouellette (1960) indicated that cell walls of vessels and ray cells, especially, were altered.

The growth of soft rot fungi and C. ulmi in inoculated, sterilized wood sections placed on agar slants, produced cavities of various lengths in secondary cell walls of woody tissue. These cavities arose from the action of microhyphae of 0.2-0.6 microns in diameter. The microhyphae grew from hyphae in adjacent cell lumen, becoming bifurcated and branched within the cell wall, or grew across the cell wall into adjacent cell lumen. The cavities resulted from the degradation of the cell wall material around the microhyphae (Casagrande and Ouellette, 1971).

The phloroglucinol test showed that no lignin occurred in the walls of newly formed cells of healthy elms. The first positive reactions for lignin occurred first in the middle lamella of the walls of parenchyma cells about mid-July, and later in secondary walls. The June examination of diseased elm, inoculated five days earlier, showed lignin
in walls of summer vessels and middle lamella of spring and early summer elements. Lamination of cell walls was more evident in diseased tissue than in healthy tissue. Thickening of walls of diseased vessels appeared to be partly due to swelling of pectins (Gagnon, 1967a).

Lignin, an end-product of metabolism, is considered to be the stiffening component of the cell wall once it is formed. It is an important substance that impregnates the wall after initial development (Frey-Wyssling, 1959). Lignin is found in the primary and secondary wall layers, as well as in the middle lamella. Lignification has been reported to begin in the primary wall adjacent to the corner thickenings of the middle lamella, from where it continues to spread to the middle lamella and the rest of the primary wall (Wardrop and Bland, 1959). Other investigators (Wardrop, 1963, 1964, 1965; Berlyn, 1964; Berlyn and Mark, 1965; Larson, 1969) suggested that lignification began in the middle lamella in cell corners and moved centripetally through primary and secondary walls. Lignification usually began sometime after deposition of the carbohydrate component of the wall (Wardrop, 1957; Berlyn, 1967).

Ultraviolet spectroscopy studies (Ruch and Hengartner, 1960; Lange, 1962) demonstrated that the highest concentration of lignin was found in the middle lamella. The concentration decreases in the secondary wall layers at
definite rates. The S2 layer contained from 18 to 25% lignin and the S3 layer contained from 11 to 18%. More than half of the lignin content occurred in the S2 layer where the concentration was lowest but the cell wall volume was greatest. Even though the lignin concentration was the greatest in the middle lamella, this area was so small that only about 10% of the total lignin could be contained within this region (Panshin and de Zeeuw, 1970).

The previous discussion on lignin, indicating early deposition of lignin in elm disease interaction followed by lignin deterioration, does not correlate with the hypothesis that the chemical pathway to lignin was shunted to phenol and polyphenol production in the host reaction in wilt diseases (Davis and Dimond, 1954). Also, little has been said about the maturity of the vessels at the time of disease initiation.

Deterioration of cellular organelles in diseased tissue has been reported recently for rice infected with Cochliobus miyabeanus (Ito and Kurib.) Drechs. Vacuolization of the nucleus was observed in infected cells. Numerous small vesicles developed within the area of the nuclear membrane (Akai et al., 1971).

Generally, the appearance of chloroplasts of healthy rice leaves was similar to those found in other higher plants. However, approximately 50 hours after inoculation of
coleoptile leaves with *C. miyabeanus* there was a change in chloroplasts close to the diseased area. These changes included vacuolar degeneration of the chloroplast. The outer-most sac of grana stacks of the chloroplast swelled into vesicle-like structures and caused layers of subunits to separate from each other. In advanced stages of chloroplast degeneration, large vesicles were changed into numerous smaller ones (Akai et al., 1971).

The mitochondria of diseased rice leaf coleoptiles also showed structural changes. Both stroma and cristae were reduced markedly, also there was appearance of vacuolar degeneration (Akai et al., 1971).

The vacuole is one of the most characteristic features of the differentiated plant cell; however, it is also the part of the cell receiving the least attention (Esau, 1963). Vacuoles were thought to be derived from the endoplasmic reticulum (Buvat and Mousseau, 1960; Poux, 1961); a process of separation from the protoplasm (Mühlethater, 1960); or from the swelling of the intramembraneous space of outer Golgi cisternae (Marinos, 1963). Vacuolar primordia have been associated with actively growing tonoplasts (Manton, 1962). Whatever may be the origin of vacuoles, they are sharply defined in well fixed material.

Many studies (Boyer and Isaac, 1964; Dufrénoy, 1932, 1936; Bailey, 1954; Bosshard, 1968; Esau, 1963; Wardrop
and Cronshaw, 1962) have indicated that polyphenols and tannins were formed within vacuoles. A recent fine-structural study on diseased elm tissue showed phenolic compounds in vacuoles of xylem parenchyma and expanded tyloses (MacDonald, 1970).

Investigations by Wardrop and Cronshaw (1962) demonstrated that in addition to phenolics being formed within vacuoles, some of these substances appeared to originate within structures limited by a double membrane. These structures resembled those of amyloplasts in form and dimensions. In younger material, adjacent to the cambium, phenolic compounds were observed in vesicles enclosed within an organelle that resembled a chloroplast with the normal structure disrupted. Further observations indicated that these vesicles arose by the dissolution of starch grains within the modified chloroplast or amyloplast. As this dissolution took place the phenolic compounds were deposited around the periphery of the newly formed vesicle until the entire structure became filled with the phenolic substances. In advanced states the modified chloroplasts or amyloplasts disintegrated and large amounts of phenolic material were deposited at the periphery of the vacuoles (Wardrop and Cronshaw, 1962).
Xylem discoloration

The discoloration of vascular tissue in wilt diseases has been widely recognized. This "browning reaction" in the conductive elements and adjacent parenchyma cells has been attributed to the formation of phenolic substances following fungal invasion of the tissue (Davis and Dimond, 1954; Winstead and Walker, 1954; Patil et al., 1962; Sequeira, 1963; Mace and Wilson, 1964; and MacDonald, 1970). Phenols have been suggested as normally functioning in defense mechanisms by combining with gels to form permanent gum plugs, or by directly inhibiting the growth of pathogens (Gordon and Paleq, 1961).

Although the means by which the fungus induces this "browning reaction" is unknown, it has been suggested that this discoloration within xylem parenchyma is produced by oxidation of phenolic compounds (Dimond, 1955). The phenols found within the vascular sap of diseased plants could arise from the action of hydrolytic enzymes, produced by the pathogen, on the native conjugated phenols that are present in the host (Davis et al., 1953; Waggoner and Dimond, 1956). Then, phenoloxidases produced by the host, could oxidize these free phenols to pigmented products.

Vascular discoloration is a prominent symptom in 
Fusarium wilt of tomato. The liberation of phenols in the host vascular system by the pathogen, 
Fusarium oxysporium f.
lycopersici probably precedes discoloration (Davis et al., 1953). Data from experiments in which free phenols are injected directly into the plant suggested that dilute phenols produce discoloration only for a short distance from the point of entry. A concentrated solution (1000 ppm) of phenols caused discoloration that extended into petioles and growing points of the plant (Davis and Dimond, 1954).

Successive microscopic observations on discolored tissue after the death of infected cells, showed that the continuous deposition of the phenolic compounds and other materials resulted in deepening of the brown color and gelatinization of the cell contents (Tomiyama, 1955). The mycelium of *Verticillium* became dark after maturity in the *Verticillium* wilt diseases. This old mycelium in vessels probably contributed to the dark discoloration of vascular bundles (Rudolph, 1931).

Vascular discoloration is also characteristic of wilt diseases of trees. Vessel discoloration of oak infected by *Ceratocystis fagacearum* Bretz is said to result from the presence of dark colored gums (Struckmeyer et al., 1954). The initial discoloration of the cytoplasm in xylem parenchyma of *Diospyros virginia* L., is caused by the presence of *Cephalosporium diospyri* Crandall in the xylem tissue. It was noted also that in later stages of persimmon wilt development, gums filled the lumen of outer
vascular elements and intercellular spaces giving the wood the characteristic streaked appearance (Wilson, 1963). Vascular discoloration has, similarly, been observed following invasion by *C. ulmi* (Schwarz, 1922; Wollenweber, 1927; Buisman, 1933; Clinton and McCormick, 1936; Kerling, 1955; Ouellette, 1962; Tchernoff, 1965; Gagnon, 1967; MacDonald, 1970).

This internal symptom of Dutch elm disease, discoloration of xylem tissue, may be present in the absence of external symptoms. The brown discoloration is associated with vessels and adjacent cells. The discoloration may appear as a broken or a continuous band around the periphery of the xylem tissue in twigs, branches and downward to the roots (Ouellette, 1960). However, several cases have been reported that show discoloration extending downward only to the root crown area (Wollenweber, 1927). Observations of cross-sections of elm twigs may reveal several discolored growth rings in succession or sometimes these discolored tissues may be separated by a healthy ring (Ouellette, 1960).

The typical brown discoloration in Dutch elm disease has been observed in springwood soon after inoculation. The discoloration appeared gradually to spread through the inoculated branch to reach the smaller twigs and branches approximately 15 days later when the first external wilt symptoms appeared. Generally, the fungus was observed to
precede discoloration of tissues, although the pathogen grew extensively in some areas without causing marked discoloration. The continued discoloration from one growth ring to the next was observed in some branches. Also, the disintegration of bordered pits and of cell walls occurred as infection developed (Schwarz, 1922; Wollenweber and Stapp, 1928).

The extent of the discoloration formed over two or more growing seasons indicated that the fungus was able to grow from one annual ring to others within the branches (Wollenweber and Stapp, 1928; Schwarz, 1922; Buisman, 1933). However, other observations in *U. americana* have indicated that fungus growth into new rings occurred mainly in the 1.5ths and lower bole of the tree (Banfield et al., 1947; Banfield, 1968).

Initial vessel discoloration of infected American elm tissue was shown by the yellowing of pit "membranes" (Gagnon, 1967b). Broekhuizen (1929) suggested that the next visible change took place in the living cytoplasm. The protoplasm became granular and the cellular contents, yellow and then brown. During the period before external symptom expression, discoloration appeared to be limited to living xylem cells having vacuoles containing tannin-like bodies, and discolored intertracheary pit "membranes" (MacDonald, 1970).
Gagnon (1967b) suggested that when solutions such as ethanol and buffers of different concentrations were injected into seedlings of *U. americana*, the results were similar to those produced when elms were inoculated with *C. ulmi*. The strong reaction found in later stages after injection showed that pathological discoloration was due to oxidation of phenolic compounds. This occurred either by synthesis or by degradation of existing substances.

**Development of gums and tyloses**

Gum and tylosic formation was part of the change that took place in diseased vessels during the general syndrome of wilt diseases (Beckman, 1964). Obstruction of vessels by tyloses and gums tended to limit the translocation of water and nutrients. This resulted in yellowing, wilting, and necrosis of leaves (Westerdijk and Buisman, 1929; Wollenweber, 1927; Buisman, 1932; Went, 1938; Pope, 1943). In the early stages of wilting only a small portion of the total conductive tissue had been shown to be plugged (Wilson, 1965). The vascular discoloration, characteristic of wilt diseases, may be primarily associated with dark colored gums in vessels (Struckmeyer et al., 1954), especially in the late stages of disease after the appearance of external wilting.

According to Chattaway (1949) both tyloses and gums were produced by ray parenchyma cells, and the size of
the pit aperture determined whether tyloses or gums were formed. However, when occlusion was severe, tyloses and gums were often noted together in the same vessels of oaks with oak wilt (Struckmeyer et al., 1954). Both gum and tylosis were thought often to develop simultaneously from identical causes, and being produced by parenchyma cells, their formation has been regarded as a vital process (Broekhuizen, 1929).

When vessels of elms are infected by C. ulmi a dark discoloration has been observed on the vessel walls. Dark colored substances were observed in xylem parenchyma and adjacent ray parenchyma. Gum droplets were excreted through pits of adjacent parenchyma, and tyloses grew into the vessels (Schwarz, 1922; Wollenweber, 1927; Wollenweber and Stapp, 1928; Buisman, 1928; Broekhuizen, 1929; Kerling, 1955; Gagnon, 1967a, b). Although tyloses were observed soon after inoculation with C. ulmi, gums were not seen in vessels during the first two-week period after inoculation in the fine-structural study by MacDonald (1970).

Pope (1943) observed the formation of tyloses within diseased elm vessels 1-3 hours after inoculation and small masses of gum within 24 hours. He suggested that the initial toxic material produced by the fungus only damaged cells and promoted tylosic formation. But later, when the toxic material increased in concentration, cell
membranes became ruptured allowing gums from cells bordering the vessels to protrude into the vessel lumen. Contrary to the finding of Broekhuizen (1929), he found that gums as well as tyloses were formed within the vessels after injection of fungal extracts. This difference may be due to the amount of extracts applied since high toxin concentrations have induced only gummosis (Kerling, 1955).

Broekhuizen (1929) also injected elms with different chemical substances such as hydrochloric acid, citric acid, oxalic acid, sodium hydroxide and ammonium hydroxide. He found that chemicals of a low pH had the highest activity in inducing gums and tyloses.

The gum that is produced in Dutch elm disease was more or less granular, varying in color from yellow to brown. It originated from xylem and ray parenchyma cells, and was extruded through pits into vessels (Broekhuizen, 1929). The variation in color was interpreted as a gradual transformation of the gum. Other reports indicated that the first changes to occur after inoculation were discoloration of vessel walls and appearance of dark contents within living cells. These substances, initially yellow, became brown gum droplets that later united into large masses, before being extruded through pits into the vessels (Kerling, 1955).

The association of gums and gels with vascular infection
has been acknowledged in a wide variety of host plants (Melhus et al., 1924; Betancount et al., 1943; Esau, 1948; Ludwig, 1952; Struckmeyer et al., 1954; Beckman et al., 1962a). According to many investigators the gum could originate from the alteration of cell wall components, especially those of a pectic nature (Ludwig, 1952; Gothoskar et al., 1953; Scheffer and Walker, 1953; Winstead and Walker, 1954; Dimond, 1955; Pierson et al., 1955; Waggoner and Dimond, 1955; Scheffer et al., 1956; Gagnon, 1967a). In addition, Butler (1911) indicated that gummosis in citrus and stone fruits resulted from dissolution of the middle lamella and primary cell wall. The brown discoloration and granular appearance of the gum itself might be due to the oxidation and formation of polymerized phenols that form melanoid substances when they combine with proteins and become trapped as gums in vessels (Dimond, 1955).

Studies on possible toxic compounds produced by C. ulmi suggested a glycoprotein (Salemink et al., 1965; Rebel, 1969). A purified form of this glycoprotein induced tylosis and gums similar to that found in elm stems after inoculation with C. ulmi spores. This toxic substance was thermostable without pectinase or cellulase activity. These findings are contradictory to those that suggested pectic enzymes produced by the pathogen were the primary cause of gummosis.
Although several investigators have discussed the development of tyloses (Haberlandt, 1923; Klein, 1923; Zycha, 1948; Chattaway, 1949; Block, 1952; Waggoner and Dimond, 1956; Stewart, 1966; Meyer, 1967; MacDonald, 1970), little critical research is available on the basic factors associated with tylosic formation.

The presence of tyloses in vessels as a natural phenomenon was observed in chestnut wood as early as 1675 (Gerry, 1914). Since that time, tylosic formation association with natural aging of tissue and with diseased tissue has been observed in the vessels of numerous vascular plants (Gerry, 1914; Chattaway, 1949; Esau, 1965; MacDonald, 1970; Sachs et al., 1970).

Tyloses are formed within vessels as intercellular growths from adjacent parenchyma cells. They may develop secondary cell wall thickening thereby resembling walls of normal plant cells (MacDonald, 1970).

Tylosic formation tended to occur rapidly within diseased elm tissue. In several cases, tyloses were observed in vessels within one day after inoculation. The initial tyloses occurred in vessels adjacent to those vessels containing fungal material. A greater number of vessels became occluded with tyloses as infection progressed. However, only a few vessels were associated with tylosic formation in the resistant selections (U. pumila L.)
var. Chinkota and _U. carpinifolia 'Christine Buisman,'), the area of tyloses remaining somewhat constant five days after inoculation. In the susceptible elm (_U. americana_) there was nearly twice the vessels area involved in tylosic formation five days after inoculation (MacDonald, 1970). Tyloses were observed both in discolored and non-discolored vessels where mycelium appeared to be absent. The tyloses formed faster in large than in small vessels (Ouellette, 1960).

Most studies at the ultrastructural level associated with tylosic development were concerned primarily with the mature wall of the tylosis. Several investigations of hardwoods have shown that these walls contain both primary and secondary layers (Foster, 1964; Koran and Coté, 1964, 1965). When two tyloses were adjacent in vessels, a structure resembling a middle lamella was observed (Sachs et al., 1970; MacDonald, 1970).

The protective layer (layer adjacent to cell wall in parenchyma cells) was observed to give rise to the tylose wall and after tyloses form, the tylose wall and the protective layer in the originating parenchyma cell were continuous (Meyer and Coté, 1968). Wound tyloses in _Quercus alba_ L. and the parenchyma cells from which they were derived were continuous and were bounded by a common cell wall (Meyer, 1967). Similar observations were made
by other workers on Eucalyptus (Foster, 1964) and oak wilt diseased tissue (Sachs et al., 1970).

An extensive electron microscopy investigation of diseased elm xylem tissue has revealed much information concerning the developing tylose. Observations indicated that both vasicentric and ray parenchyma were capable of producing tyloses. Vessel-parenchyma pit "membranes" were the site of initiation for tylosic formation. A protective layer was observed in most xylem parenchyma. Normally this layer extended around the inside periphery of the parenchyma cell, being much thicker near the vessel-parenchyma pit "membrane." The pit "membrane" was considered to be a flexible structure because no rupturing was observed during expansion of tyloses into the vessel lumen. The protective layer appeared to expand by the addition of material from the cytoplasm (growth) (MacDonald, 1970).

At maturity, walls of the tyloses were composed of a normal middle lamella, a primary wall and a secondary wall. After the expansion of the full tylose the protoplast reverted to synthesizing secondary wall material (Foster, 1964; MacDonald, 1970). This wall material was deposited inside the protective layer within the tylosis within the vessel and within the originating parenchyma cell.

Small vacuoles, that appeared to be bounded by membranes, were observed in the cytoplasm as the tyloses enlarged.
Vacuolation was noted also in parenchyma cells from which the tyloses originated. The cytoplasm usually was suppressed against the cell wall by the large vacuole that fills the tylosis, at maturity of the tylosis (Wardrop and Cronshaw, 1962; MacDonald, 1970).

Organelles of tyloses and those of the parenchyma cells from which they originated were similar. In most cases, the cytoplasm contained endoplasmic reticulum, ribosomes, numerous dictyosomes, and many vesicles (MacDonald, 1970).

Possible Bases for Host Resistance

Various factors have been studied in relation to host resistance to vascular wilt diseases. The most promising suggestions in Dutch elm disease include production of phenolic compounds, growth of tyloses, and xylem anatomy.

Production of phenolic compounds

That certain aromatic compounds play a role in resistance has been widely believed. Phenolic substances, such as phenolic glycosides, flavonoids, and coumarin derivatives have been found to increase in host tissue invaded by pathogens (Kuć, 1963). In most cases, an increase in these phenolic compounds was more rapid in the resistant tissue than in susceptible tissue (Goodman et al., 1967). The role of such phenols has been thought to be diverse, with their ability to undergo oxidation being important (Manskaya,
The function of phenolic compounds in plant tissue has been suggested as one of warding off or restricting the attack of pathogenic organisms (Cook and Taubenhaus, 1911; Byrde, 1956, 1957; Cook and Wilson, 1961; Williams, 1963). In cases where the progress of a pathogen was checked, phenolic compounds, mainly those of the gallic group, developed in abundance within vacuoles of cells in advance of cell infection (Dufrênoy, 1932, 1936). However, when a pathogen thrived within host tissue and caused systemic infection, phenols formed slowly and in very small amounts.

The accumulation of phenolic compounds is considered an integrated process that includes taking into consideration, a three-dimensional, geometric view of the infection site and adjacent healthy tissue. In other words, the role of common phenolic compounds in disease resistance can only be evaluated from the viewpoint of a dynamic response of the healthy cells that surround the infection site (Tomiyama et al., 1967).

Dimond (1955) has summarized two hypotheses on the possible mode of liberation of phenolic compounds. Phenols that could be liberated from specific glucosides through B-glucosidase and synthesized into lignin in normal plants, might be oxidized to melanin-like substances in diseased plants. Or, phenols might originate as a result of B-
glucosidase action on a number of glucosides independently and these oxidized in a similar manner.

Phenolic compounds could act upon fungi in various ways. They could inhibit fungal growth or could reduce production of, or restrict the activity of exoenzymes that degrade host tissues and provide for nutrition of the pathogen (Waggoner and Dimond, 1956; Deese and Stahmann, 1962).

Much research concerning the activity of phenolic compounds has been conducted with potato tuber discs infected with Phytophthora infestans (Mont.) de By. This work has produced a number of basic concepts relating to the action of phenols in disease resistance: the need for high metabolic activity in living cells near the infection site (Sakuma and Tomiyama, 1967); continuous flow of phenols to infection site from adjacent living tissue (Tomiyama et al., 1967); and confinement of the pathogen to the necrotic tissue near infection site by the action of the oxidation products of the phenols (Williams, 1963). This necrotic area became discolored and the overall reaction likened to a hypersensitive reaction (Tomiyama, 1955).

Identification of specific phenolic substances thought to be associated with resistance has been attempted. Examples are coumarin, scopoletin (Best, 1936, 1944), arbutin (Strobel and Mathre, 1970), chlorogenic acid (Strobel and Mathre, 1970), phlordanz (Barnes, 1960; Barnes and
Williams, 1961) and 0-quinones (Hampton and Fulton, 1961). In woody plants, a heterogenous group of phenolic derivatives of high molecular weight, the tannins, usually has been associated with disease resistance considerations. These substances have appeared in vacuoles at their inception, or in the cytoplasm as small droplets that eventually fuse. Tannins have caused difficulty in fixing material for electron microscope examination. Bodies of this material have a wavey surface appearance in cross-section because of their hardness in cutting. Such material in mass might also have many clear spaces like holes; these were assumed to be areas where material was lost in the fixing and sectioning process (MacDonald, 1970). Cells containing much tannin have been commonly found adjacent to cells free of this material (Esau, 1963). Tannins have occurred widely and frequently in high concentration in plants infected by fungi. The ultimate toxicity of tannins to pathogens, though, would depend, in part, on the type of phenolic or other toxic substances formed by the interaction of pathogen and host tannin (Offord, 1940).

Tannins have been thought to protect the protoplast against desiccation, decay and injury. They also have been interpreted as reserve substances related in some undetermined manner to starch metabolism; as substances associated with sugar formation and transport; as anti-
oxidants; and as protective colloids maintaining the
homogeneity of the cytoplasm (Esau, 1965).

The term "tannin" has been used to describe numerous
phenolic derivatives, but presently, this group of compounds
is referred to as "polyphenols." These polyphenols have
been found normally in most tissues of woody plants. They
have been associated with the formation of pigmented
products in diseased banana roots (Mace, 1963), natural
heartwood formation (Bailey, 1954; Bosshard, 1968), and gums
in vessels and parenchyma cells of oak wilt diseased tissue
(Elgersma, 1967; Sachs et al., 1970). Such xylem discolor­
atation associated with polyphenols has been found commonly
in Dutch elm disease (Gagnon, 1967a; MacDonald, 1970).
Injury-induced polyphenols, including those induced by
disease have appeared at the same sites of accumulation as
in normal heartwood formation, but seemed to differ chemically
(Hillis, 1968).

The bark of many woody plants has been observed to con­
tain tannin and other complex polyphenols (Klopping and
van der Kerk, 1951). Some of these tannins (pyrocatechol,
salicin, saligerin and salicylic acid derivatives) have
been reported to possess fungistatic activity. Although
tannins have been suggested as playing a role in resistance
of many plants, the presence of different types in the bark
of three species of chestnut (Castanea sp.) was not a
factor in relative resistance to the chestnut blight fungus. Instead, it was suggested that the variation in resistance of the three species was, at least in part, due to the differences in solubility and amount of pyrogallol and catechol tannins present (Nienstaedt, 1953).

Glucose labelled with $^{14}C$ has been used to study the production of polyphenols in *Eucalyptus sieberiana*. These studies suggested that the formation of polyphenols was derived from sugars. *E. sieberiana*, collected within a few days after growth and normal phenolic formation began, was composed almost entirely of polymeric polyphenols (Hillis and Hasegawa, 1963).

The previous electron microscope study of initial elm host response to *C. ulmi* indicated the accumulation of oxidized phenolics in xylem parenchyma and developing tyloses (MacDonald, 1970). This research also indicated two distinct types of phenols during the sequence of host reaction from inoculation to external symptom expression. Early, a fine granular material was seen accumulating in vacuoles of parenchyma cells. This material was probably not visible until stained with osmium tetroxide in the process of preparing the material for observation. This material could be the phenols before oxidization. Later, tannin-like bodies, difficult to section and similar to naturally occurring tannins, were observed in cell
vacuoles. These were interpreted as oxidized phenols and part of the discoloration seen in diseased elm branches. These host reactions were found in a reactive zone caused by fungus invasion. This zone was much more limited in area in resistant elms than that found in the susceptible American elm.

**Growth of tyloses**

Tyloses have been observed in vessels of many plants (Chattaway, 1949; Esau, 1965). Their production has been considered a natural part of aging and the formation of heartwood in some species (Esau, 1965). Abnormal tyloses have formed upon wounding (Meyer, 1967), water stress (Zycha, 1948), and presence of disease organisms (Struckmeyer et al., 1954; Beckman et al., 1962b; Tchernoff, 1965). Literature on the nature and structure of the tylosic growth has been reviewed in an earlier section.

Tylosic formation has been another characteristic of the reactive zone in Dutch elm disease (MacDonald, 1970). The blockage of vessels by tyloses could limit the vertical distribution of the pathogen (McNabb et al., 1970). The rapidity of tylosic production would determine the effectiveness of vertical blockage (Beckman, 1966; Brener and Beckman, 1968). Although the reaction zone was larger in area in a given period of time in a susceptible host, no difference among elm selections was seen in initial time
required for tylosic growth (Elgersma, 1969; MacDonald, 1970).

**Xylem anatomy**

The period of highest susceptibility of *U. americana* to Dutch elm disease has been correlated with high cambial activity and springwood formation (Lodewick, 1928; Thompson, 1954; Pomerleau, 1966). The springwood vessels and their arrangement in American elm have been thought conducive to rapid distribution of the fungus; vessels segments having large diameters and lengths, and many interconnections among vessels (Pope, 1943).

Differences in anatomy among elm species were first noted by Pope (1943). Spring vessels were smaller and interconnections less when *U. pumila* was compared with *U. americana*. Size and grouping of vessels at the beetle feeding site, in two-year-old twigs, were studied in a number of elm selections representing a wide range of reaction to Dutch elm disease (McNabb et al., 1970). Increasing susceptibility correlated highly with increasing vessel group size (product of average vessel diameter and the number of contiguous vessels). Longer vessel segments also correlated with higher susceptibility (Elgersma, 1970).

If localization of the pathogen in host tissue is a means of host resistance, xylem anatomy could contribute to the effectiveness or noneffectiveness of phenolic
compounds and tylosic production in limiting the distribution of the pathogen in the elm host (McNabb et al., 1970). The reaction zone became a visual measure of this limited or nonlimited distribution, both in gross observations of discoloration (Kerling, 1955; Tchernoff, 1965) and microscopic examination of disrupted tissue (MacDonald, 1970).
MATERIALS AND METHODS

Host Material Used in the Study

The two field-grown elm selections used in this study were chosen because their reaction to Dutch elm disease was known. All trees used were located on the Old Iowa State University Horticulture Farm on South Hayward Avenue, Ames. The susceptible *Ulmus americana* (Figure 1) and the resistant *U. carpinifolia* Gleb. 'Christine Buisman' (Figure 2) were used. *U. carpinifolia* 'Christine Buisman', an early Dutch clonal selection, (Clone 24 from Madrid, Spain) was the first resistant Dutch selection released, in 1936, from their breeding program. This study was initiated during the late spring of 1969. The trees used in this experiment were similar in age and diameter at breast height.

Inoculum

The inoculum was obtained from fourteen-day-old mixed shake cultures of five isolates of *C. ulmi*. The cultures were grown on 50 ml of a modified Zentmeyer's liquid medium contained in 125 ml Erlenmeyer flasks (Ouellette and Gagnon, 1960). The five fungal isolates were obtained from recent isolations of *C. ulmi* taken from *U. americana*.

The contents of three to four shake-culture flasks were mixed together and divided into large centrifuge tubes with the excess spore suspension being discarded. The
Figure 1. *Ulmus americana*, American elm, single field grown tree
Figure 2. Ulmus carpinifolia 'Christine Buisman', Buisman elm, the Dutch clonal selection #24 from Madrid, Spain
tubes were centrifuged at 3,000 g for 10 minutes at room
temperature. After decanting the supernatant, the spores
were washed three to four successive times with sterile
distilled water. The same centrifuging procedure was
followed after each washing. The spore pellets were diluted
with 100 ml of sterile, distilled water. The spore concen-
tration of this suspension was determined within 5 minutes
after dilution; and, the suspension was used within 15
minutes thereafter for inoculation. The conidial suspension
contained approximately $10^4$ spores per ml.

Inoculation and Sampling

Two-year-old twigs were selected from each host for
inoculation. This age was selected because it represented
the age tissue preferred by the smaller European elm bark
beetle (Scolytus multistriatus Marsh) for its feeding
activity. A droplet of spore suspension was placed on the
twig at the point of inoculation. A dissecting needle was
used to prick the twig to a depth just below the cambium
(Figures 3, 4). The inoculum was drawn into the twig as
the needle was removed. Control twigs were inoculated in
a similar manner with sterile distilled water.

On June 21, 1969, a total of 60 twigs, 30 from each
tree selection, were inoculated at random. Collections
of inoculated twig material from U. americana were made
at 11, 14, 17, 20, 40, and 43 days after inoculation.
Figure 3. Inoculating a two-year-old elm twig

Figure 4. Point of inoculation tagged for future collection
At each collection period, four replications of inoculated twigs and a single control twig were collected for each of the tree selections (Table 1). Tissue to be studied was taken from at least 3 inches above and below the inoculation sites where pronounced sapwood discoloration was present.

Two to three twig segments were removed from both distal and basal ends, at least 3 inches from the point of inoculation. A portion of each twig segment was preserved in FFA solution (Sass, 1958) for later thick sectioning, light microscope examination and tests for the presence of lignin. The other twig sections to be used in light (thin section) and electron microscope studies were immediately transported to the laboratory for fixation and embedding.

The area of the twig to be studied was transversely cut with a sharp razor blade. Subsequent transverse disc sections were cut approximately 1/32 to 1/16 inches thick. These sections were fixed for one hour at 4°C in 4 ml, 4 per cent gluteraldehyde and 2 ml, 2 per cent acrolein mixed with 44 ml phosphate buffer.

The procedures used for dehydration and the embedding process of Araldite-Epon (Anderson and Ellis, 1965) are found in Appendix A. Material collected on all sampling dates was embedded in Araldite-Epon.
Table 1. Times and replications for inoculation and collection of two-year-old elm twigs

<table>
<thead>
<tr>
<th>Selections</th>
<th>Date of inoculation</th>
<th>No. of inoculated twigs collected on each date</th>
<th>No. of control twigs collected on each date</th>
<th>Day collected after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>U. americana</em></td>
<td>June 21, 1969</td>
<td>30</td>
<td>4</td>
<td>11, 14, 17, 20, 40, and 43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38</td>
</tr>
<tr>
<td><em>U. carpinifolia</em></td>
<td>June 21, 1969</td>
<td>30</td>
<td>4</td>
<td>11, 14, 17, 20, 46, and 76</td>
</tr>
<tr>
<td>'Christine Buisman'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total twigs collected</td>
<td></td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Control twigs were inoculated with sterile, distilled water.
Test for the Presence of Lignin

Two-year-old twig cross-sections 20 to 30 microns thick, previously fixed in FAA and thoroughly washed in 50% ethanol, were placed on pre-cleaned microscopic slides. A drop of phloroglucinol-ethanol solution was added. After allowing part of this solution to evaporate, a drop of 25% hydrochloric acid was added and a coverslip placed over the tissue. Observations were made after 10 to 20 minutes. A bright red color denotes the presence of lignin (Johansen, 1940).

Light and Electron Microscopy

Sections, 1 to 2 microns thick, embedded in Araldite-Epon, were prepared for light microscopy observations. These sections were cut with a glass knife on a LKB ultramicrotome. Electrical tape and dental wax were used to modify the glass knives. The purpose of this was to provide a water surface upon which sections were floated. The sections were picked up with a loop made from baby hair and placed on the end of a capillary tube. The sections were placed on a slide in a drop of distilled water. The slide was heated to aid drying. The sections were mounted permanently in piccolyte and observed by phase-contrast light microscopy. Aqueous safranin O was used to stain additional slides for permanent mounting (Feder and O'Brien, 1968). This staining procedure was used to enhance contrast
for photographic purposes.

Light microscopy observations were important in this study. After an area of interest was observed at the light level, the specimen was trimmed to include these desired cells, and additional sections were prepared for electron microscopy.

Photographs of light microscopy observations were taken on a Zeiss photomicroscope using an automatic 35-mm camera. Bright-field photographs were taken on Kodak Panatomic-X film.

Sections, embedded in Araldite-Epon material, for electron microscope studies were cut with a Dupont diamond knife on an LKB Ultratome III ultramicrotome. The thickness of desired sections ranged from 60-90 millimicrons. They were selected by observing the color (silver to light gold) of the reflected light. A pair of fine pointed forceps was used to collect sections on 100 or 200 mesh carbon-coated, formvar-supported copper grids. Thin sections were stained with methanol uranyl acetate (Stempak and Ward, 1964).

An RCA EMU-3F electron microscope operated at 50 kv, was used to observe all specimens. Direct magnifications of approximately 3,500 to 15,000 x were photographed on Cronar Ortho-s-Litho film. These films were developed in Kodak D-19 developer for 1.5-2.0 minutes at 68° F. All
negatives were enlarged and printed on Kodabromide F single-weight paper.
RESULTS AND OBSERVATIONS

The results obtained in this study were based on host reactions after the appearance of external symptoms. The deterioration of host tissue, formation of tyloses, accumulation of phenolic-like or tannin-like material, and the formation of gum-like material were observed in both elm species. An attempt will be made to discuss both light and electron microscopic observations, simultaneously.

Deterioration of Host Tissue

This phase of the study was implemented more at the electron microscope level. Although it was possible to observe some cellular deterioration within a few parenchyma cells at the light level (Figures 13, 14, 16, 17, 18, 20), it was difficult to make detailed studies of cellular organelles.

Although observations were made during a time sequence, initial symptom expression (11 days after inoculation) to advanced symptom expression (43 days after inoculation), in U. americana, all gradations of deterioration could be seen at any level of symptom expression. Therefore, much of the examples of American elm presented will have little time sequence meaning. On the other hand, the host reaction in the Buisman elm was more intense and localized than in American elm. Therefore, there appeared to be more rela-
tionship between time sequence and tissue deterioration in this elm species.

During initial stages of disease the major organelles (Figures 7, 8, 10, 11) of reacting parenchyma cells, such as the nucleus, the chloroplast-like structures and mitochondria appeared near-normal (Figures 26, 63), with some chloroplast-like structures containing starch grains (Figure 63). However, in advanced stages of the disease most organelles became distorted. The cristae of mitochondria appeared to separate from the inner membraneous structures and to form small vesicles (Figures 30, 32, 35, 37, 39, 42, 46, 49, 63). In several parenchyma cells normal mitochondria were observed along with distorted ones (Figures 32, 37). In addition, tyloses also contained distorted mitochondria (Figures 30, 46). Disrupted chloroplast-like structures often gave rise to vesicles (Figures 27, 28).

The deterioration of the nucleus was observed in several parenchyma cells. One type of deterioration appeared to be associated with the breakdown of the membrane structure (Figures 31, 39, 40, 50, 61). Another type of nuclear deterioration was the separation of the double nuclear membrane (Figures 27, 29, 42, 62, 63). Vesicles formed in the area of the nuclear membrane separation (Figure 29).

During the disease reaction parenchyma cells, both ray and vasicentric, became highly vacuolated (Figures 27, 30,
32, 35, 42, 44, 49, 56, 59). Vesicles originated from the breakdown of the plasmamembrane (Figures 26, 27, 28, 29, 32, 35, 37, 42, 63). After formation these vesicles moved toward the center of the cell. In some cases, vesicles seemed to pinch off from dictyosomes (Figures 32, 35, 62). As indicated before, vesicles were noted to form from disrupted areas of chloroplast-like structures (Figures 27, 28). Other membrane systems, associated with endoplasmic reticula, also aided in the formation of vesicles (Figures 27, 28, 29, 35, 37, 42). Vacuoles and vesicles were especially noticeable in several tyloses (Figures 42, 44, 46, 50). Phenolic-like and tannin-like substances accumulated in vacuoles of ray and vasicentric parenchyma cells (Figures 27, 35, 36, 37, 38, 42, 47, 56, 60, 62, 63).

Numerous starch grains were observed in both healthy and diseased parenchyma cells (Figures 7, 9, 11, 12, 26, 34, 35, 36, 37, 38, 40, 41, 44, 47, 55, 56, 57, 58, 61, 62, 63). Some starch grains appeared abnormal (Figures 39, 41, 44). In some diseased and apparently dead cells, starch grains were the only structures that remained (Figures 34, 36, 55). In addition, cells containing tannin-like material in vacuoles also contained starch grains (Figures 35, 36, 37, 38). Starch grains were observed in tyloses (Figures 44, 49, 60).

Although killing and fixing procedures were the same
for all samples, "tearing" in the middle lamella area of the cell wall was noted in a few advance diseased samples (Figures 31, 59, 60, 61). I believe this indicated deterioration of the cell wall, whether the presence of the "tear" was from the condition of the original tissue or from poor killing and fixation. Since, in general, the prepared material gave good results, cell wall "tearing" caused by poor killing and fixation could indicate deterioration at that point. If this area was the condition of the original tissue, it could be compared with the cavities described by Casagrande and Ouellette (1971), and caused by the degrading of wall material by *C. ulmi*. The dark staining area in the cell wall near the "tearing" (Figures 59, 60, 61) and the round, dark staining areas in the cell walls (Figures 57, 58) could be interpreted as microhyphae (Casagrande and Ouellette, 1971).

A definite layered appearance of the secondary cell walls was observed commonly in advanced diseased tissue (Figures 28, 29, 31, 56, 57, 59, 60, 61, 63). Fainter layers were seen in noninfected tissue (Figures 7, 8, 10). These layers appeared to be similar to the S layers described for secondary cell walls of woody tissue (Panshin and de Zeeuw, 1970).

Degree of lignification of the cell walls was determined with the phloroglucinol test. The walls within the diseased
area reacted to produce a deep red color signifying heavy lignification. Normal tissue walls appeared reddish but this color was not nearly as intense as that seen in diseased tissue. In addition, some vessel walls of early diseased tissue became yellow to gold in color using the phloroglucinol test.

Parenchyma cells were found without secondary cell walls. Such cells were located adjacent to the cambium (Figure 32) and among cells having secondary walls (Figure 57).

Formation of Tyloses

Initiation of tyloses occurred in the diseased area of most host tissue (Figures 13, 14, 15, 16, 19, 20, 22, 23, 24, 25, 29, 30, 37, 42, 43, 44, 45, 46, 47, 50, 51, 53, 54, 57, 59, 61). Where several tyloses occupied a vessel, the area of open vessel was reduced (Figures 13, 15, 16, 25, 43, 44). Some tyloses contained vacuoles (Figures 30, 42, 44, 46, 50), disrupted mitochondria (Figures 30, 46), and phenol-like material (Figures 42, 44). Tyloses often developed to contacting each other and formed adjacent cell walls similar to those between developing normal parenchyma cells (Figures 14, 15, 30, 44), including simple pits (Figure 23).

Occasionally, tyloses formed secondary wall layers (Figures 15, 23). Whenever a tylosis formed this secondary wall, the parenchyma cell from which it grew, produced a second-secondary cell wall layer (Figures 29, 31, 36, 56,
57, 62, 63) continuous with the secondary wall in the tylosis (MacDonald, 1970). This new secondary wall was separated from the first by a thin discolored line, probably representing the original protective layer.

Discoloration of Xylem Tissue

Although the electron microscope provided more detailed observations of the probable sources of discoloration in diseased tissue, the light microscope was useful in presenting a general view over a wider area of tissue.

The intertracheary pit "membranes" in most diseased tissue appeared discolored (Figures 13, 14, 15, 17, 19, 21, 24, 42, 44, 55, 58, 59) when compared with normal tissue (Figures 5, 6). Abnormal discoloration also appeared on the wall surface within the bordered pits (Figures 55, 58, 59) and vessels (Figures 17, 19, 20, 23, 24, 42, 55, 58, 59) of diseased tissue.

Probable sources of discoloration associated with vessel lumens were observed as phenol-like material in the vacuoles of tyloses (Figures 42, 44), free phenol-like bodies in lumens (Figures 28, 55), possible deteriorating fungus material (Figures 51, 53, 54, 55), and gum-like substances (Figures 50, 51, 53, 54, 55). In one case, gum-like or phenol-like material appeared to be moving into a vessel from an adjacent parenchyma cell (Figures 48, 49).

The apparent areas of discoloration within the parenchyma
48

cells seemed to be the tannin-like bodies within the vacuoles (Figures 27, 35, 36, 37, 38, 62, 63), the accumulation of phenol-like material within the vacuoles (Figures 42, 47, 59, 60, 61), and the phenol-like material in the cytoplasm or in place of the cytoplasm (Figures 32, 39, 40, 41, 49, 50, 51, 56). The changes to phenol-like material within the cytoplasm were observed primarily in the Buisman elm.

Observational Differences between Elm Species

Under gross observations, the resistant Buisman elm had a more localized reaction to *C. ulmi* than the susceptible American elm. This reaction appeared to be more intense in the Buisman elm, illustrated by the rapid deterioration of the cytoplasm leaving intact organelles (Figures 32, 39, 40). All gradations of apparently normal, living parenchyma cells to dead parenchyma could be observed in sections of American elm (Figures 26, 34, 63). Normally, only advanced deteriorated to dead cells were seen in sections of the Buisman elm (Figures 41, 55).
DISCUSSION AND CONCLUSIONS

The responses of xylem tissue to *Ceratocystis ulmi* in the two elm species studied, were similar in that tissue deterioration, tylosic production, and accumulation of phenol-like, tannin-like and gum-like material occurred. However, the apparent sequential deterioration of cell organelles, the production of gums, and the formation of tyloses were more noticeable in the susceptible species. The latter two characteristics were probably observed less in the Buisman elm because a much smaller area of reacting tissue was present in this resistant species than that found in the susceptible American elm (MacDonald, 1970).

During the relative slow death of most parenchyma cells in diseased American elm tissue, the cell organelles deteriorated through an observable sequence to complete breakdown. Numerous small vesicles formed within the deteriorating mitochondria and chloroplast-like bodies. Vesicles and vacuoles formed near the deteriorating nuclear membrane. These observations agree with those of Akai and co-workers (1971). In diseased Buisman elm apparent deteriorated cytoplasm of parenchyma cells, contained organelles that seemed normal to near-normal. This observation of near-normal organelles in dead-appearing parenchyma cells could be a result of quick death of these cells in the host reaction to the disease fungus. Later observa-
tions on diseased tissue of this resistant host showed parenchyma cells containing only products of deterioration, phenol-like, tannin-like or gum-like material.

The membrane systems of the cytoplasm, the plasmamembrane, dictyosomes and endoplasmic reticula formed vesicles coalescing into vacuoles in the deteriorating parenchyma cells of American elm diseased tissue. These observations support the idea that vacuoles are derived from endoplasmic reticula (Buvat and Mousseau, 1960; Poux, 1961) and dictyosomes (Marinos, 1963). I did not observe the formation of vacuoles from the separation of protoplasm as reported by Muhlethater (1960). A dictyosome was seen in the deteriorated cytoplasm of a parenchyma cell of the Buisman elm tissue. Although vacuoles and vesicles were seen in xylem tissue of this resistant elm species, the degree of formation was less than that found in American elm. Therefore, the formation of vesicles and vacuoles is associated with xylem tissue diseased by _C. ulmi_, especially that tissue of the susceptible elm whose parenchyma cells deteriorate relatively slowly.

In discussing the reaction areas in elm tissue diseased with Dutch elm disease, MacDonald (1970) suggested that parenchyma cells adjacent to vessels containing the disease fungus, _C. ulmi_, were killed quickly, while those parenchyma cells beyond the first cells reacted by forming
tyloses and slowly deteriorating. I would like to further suggest that my observations in Buisman elm of apparent rapid deterioration of parenchyma cells and deposition of the products of deterioration indicate parenchyma cells other than those adjacent to vessels containing C. ulmi are killed quickly. This could be considered a hypersensitive reaction (Tomiyama, 1955) that results in localizing the growth of the fungus in this resistant elm. The apparent rapid deterioration of the cytoplasm to phenol-like material would add to this localizing effect if such material were anti-fungal in nature.

Another feature of diseased tissue is tylosic formation. Tyloses can be found growing into vessels soon after inoculation with C. ulmi (Pope, 1943; MacDonald, 1970). Since tyloses are outgrowths of parenchyma cells into adjacent vessels, what has been indicated for slowly deteriorating parenchyma cells is also applicable to deteriorating tyloses. In addition, if the tylosis matures completely before deterioration begins, a secondary cell wall is deposited within the tylosis. I further verified MacDonald's observation that the tylose secondary wall extended into the mother parenchyma cell producing a second-secondary wall adjacent to the old protective layer (1970).

Vessels were found with several tyloses, indicating blockage of vessels by tylosic growth alone would be
possible. Therefore, tyloses could play a role in limiting the vertical distribution of the pathogen (McNabb et al., 1970).

The internal symptom, sapwood discoloration, of Dutch elm disease has been attributed to deteriorating diseased tissue and the resulting colored substances produced in this tissue breakdown. During the early period of disease before external leaf symptoms, MacDonald found a discoloration of intertracheary pit "membranes", and phenol-like material forming in the vacuoles of parenchyma cells, followed by the appearance of tannin-like bodies in these vacuoles (1970). He, therefore, indicated that the discoloration during this phase of the disease was associated primarily with the parenchyma cells and their outgrowths, tyloses. During the phase after external symptom development, I also found discolored intertracheary pit "membranes" and vacuoles of diseased parenchyma and tyloses forming phenol-like material and containing tannin-like bodies. In addition I found cytoplasm of parenchyma becoming phenol-like in appearance, phenol-like to gum-like material being deposited into a vessel from a parenchyma cell, discoloration of the surface of vessel walls, phenol-like to tannin-like material in vessels, and gum-like and apparently deteriorated fungus materials together with deteriorating tyloses in vessels. The association of vascular elements
with discoloration has been made before (Schwarz, 1922; Broekhuizen, 1929; Buisman, 1933; Kerling, 1955; Gagnon, 1967b).

In addition to discolored material, starch grains were seen commonly in diseased parenchyma cells. Such starch grains were present in parenchyma cells with phenol-like material and tannin-like bodies. In fact, many dead parenchyma cells were found containing starch grains and tannin-like bodies. These observations were somewhat contrary to the findings of Wardrop and Cronshaw (1962) and MacDonald (1970) who suggested that the metabolism of carbohydrates and phenol-like substances are interrelated. They observed parenchyma cells containing abundant phenol-like or tannin-like material to have little or no starch.

Little electron microscope observations have been made on gum-like material in diseased vascular tissue. Several workers (Pope, 1943; Struckmeyer et al., 1954; Kerling, 1955; Gordon and Paleq, 1961) have indicated that gums have a role in the discoloration of diseased xylem tissue. The gum-like material that I observed within vessel lumens appeared in three forms; masses of irregular shapes, phenol-like material being produced from adjacent parenchyma cells (Broekhuizen, 1929), and a mixture of material thought to be deteriorated fungus hyphae and gum. This latter mixture has been referred to as "gunk". The suggestion that
deteriorated fungus material could be involved in discoloration has been made before (Ouellette, 1960). The finding of this gum-like material and deteriorating tyloses together within the same vessel lumen has also been observed in the oak wilt disease (Struckmeyer et al., 1954). From my observations, I believe the gum-like materials observed in the vessels were a product of the deteriorating living cells of both the host and the pathogen. I did not observe gum-like material being formed from the degrading of cell walls (Gagnon, 1967a).

Observations made on the cell walls of diseased host tissue presented a number of questions. Distinct discoloration of the surface of vessel walls was not found commonly. Since this type of discoloration was more common on the borders of bordered pits, and such pit "membranes" were discolored under diseased conditions, I suggest a possible relationship among these discolorations.

The presence of layers in the secondary cell walls of many cells suggested that these walls were being defined into the commonly understood S layers of lignified secondary walls (Panshin and de Zeeuw, 1970). This layering was not observed in MacDonald's study (1970). In non-diseased tissue, faint layering was observed. Two explanations can be presented for the apparent high lignification of the cell walls in diseased tissue.
Gagnon (1967a) has indicated early lignification in diseased tissue. Also, my study, being on the host reaction after external symptom expression, has utilized host tissue later in the growing season than MacDonald utilized (1970). I believe my observations represent more mature cell walls because of later season sampling modified by the disease reaction suggested by Gagnon (1967a).

Parenchyma cells at all stages of maturity were found diseased. Cells adjacent to the cambium that had no secondary wall formation to highly lignified walled cells were found in all stages of deterioration from C. ulmi. I do not believe the question of cell maturity has been raised before when considering host reaction to the pathogen. Most investigators have considered that xylem tissue is mature when the vessel lumens clear and vessel cross walls deteriorate. From my observations I do not believe this to be the case. If lignification of cell walls continues and is not complete until a time during the late growing season, what other aspects of cell maturity are delayed until later in the season?

The presence of microhyphae-like structures in the secondary cell wall and middle lamella areas of diseased elm tissue seem to be similar to recent work of Casagrande and Ouellette (1971). They suggested that microhyphae are responsible for producing cavities of various lengths in
the cell walls of diseased tissue. The "tear" observed in my material can be interpreted as possible cavities produced by the degrading of wall material by microhyphae. Although poor fixation procedures could explain the "tear", the near normal appearance of tissue surrounding this area suggest something beyond killing and fixing as the cause of this "tear". Therefore, I suggest that internal wall degradation by the pathogen, *C. ulmi*, was observed in diseased material after external symptoms were expressed.

In conclusion, I feel that this study has provided another step in the determination of internal host responses associated with Dutch elm disease. The cause of discoloration of diseased tissue has been further established to be associated with the deterioration products of host parenchyma cells and tyloses, and fungus material. Additional research emphasis should be concerned with the identification of the phenol-like and tannin-like substances of deterioration, and the determination of the significance of microhyphae in wall breakdown during the active reaction phase of the disease syndrome. The question of cell maturity has been raised in this study. I believe investigations concerned with this question would prove fruitful in the determination of the differences in host cell reaction to the presence of *C. ulmi*. 
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APPENDIX A: FIXATION, DEHYDRATION, INFILTRATION, EMBEDDING AND LIGNIN TEST
**Fixation**

**Glutaraldehyde-Acrolein fixation**

4 ml gluteraldehyde (2%) and 2 ml acrolein (1%) added to 44 ml of 0.05 M phosphate buffer at pH 7.2; fixed 1 to 2 hours at 4°C.

1. Preparation of phosphate buffer (Lillie, 1954):
   
   \[
   \begin{align*}
   0.1 \text{ M } \text{KH}_2\text{PO}_4 & \quad 11 \text{ ml} \\
   0.1 \text{ M } \text{Na}_2\text{HPO}_4 & \quad 39 \text{ ml}
   \end{align*}
   \]

   Post-fix for one hour in 2% osmium tetroxide after three 20-minute washes with the phosphate buffer.

**Dehydration and infiltration**

Araldite-Epon specimens were dehydrated in the following manner at room temperature. All specimens were slowly rotated on a rotating mixer during all phases of dehydration and infiltration.

**Dehydration**

1. 5 minutes each in 25, 50, 70 and 95 percent ethanol
2. 3 changes of 5 minutes each in absolute ethanol
3. 3 changes of 5 minutes each in propylene oxide

**Infiltration**

1. 15 minutes in a mixture of 1 part Araldite-Epon:
   
   \[
   3 \text{ parts propylene oxide}
   \]

   2. 30 minutes in a mixture of 1 part Araldite-Epon:

   \[
   1 \text{ part propylene oxide}
   \]
3. 1 hour in a mixture of 3 parts Araldite-Epon:
   1 part propylene oxide
4. 12-18 hours in pure Araldite-Epon
5. Specimens were embedded in shallow boats made of aluminum foil
6. Stepwise polymerization was carried out at 37° C for 2 days, 45° C for 2 days and 60° C for 3 days

**Embedding**
Specimens were embedded in Araldite-Epon (Mollenhauer, 1964; Anderson and Ellis, 1965).

**Araldite-Epon**
1. Araldite 502 10 ml
2. Epon 812 13 ml
3. DDSA 30 ml
4. DMP-30 2 ml

**Lignin test** (Johansen, 1940)
0.1 g phloroglucin in 10 cc of 95% ethanol
25% hydrochloric acid
allow part of the phloroglucin-ethanol solution to evaporate, then add a small drop of hydrochloric acid
APPENDIX B: KEY TO LABELLING
Ca  Cambium
Cl  Chloroplast-like structure
CW  Cell wall
D  Dictyosome
ER  Endoplasmic reticulum
G  Gum-like material
IM  Intertracheary pit "membrane"
L  Cell wall layers
M  Mitochondrion
MH  "Microhypha"
ML  Middle lamella
N  Nucleus
NM  Nuclear membrane
P  Phenolic-like material
Pd  Plasmodesmata
Pl  Protective layer
Pm  Plasmamembrane
S  Starch grain
SW  Secondary cell wall
SSW  Second-secondary cell wall
T  Tylosis
TM  Tannin-like material
V  Vacuole
VL  Vessel lumen
VS  Vesicle
APPENDIX C: FIGURES
Figure 5. Cross-section of *U. americana*, from control sample. Note the large vessels and abundant starch grains within ray and vasicentric parenchyma cells. Line scale represents 5 microns.

Figure 6. Cross-section of *U. carpinifolia* 'Christine Buisman', from control sample. Note many small vessels and the few starch grains in parenchyma cells. Line scale represents 5 microns.
Figure 7. *U. americana*, from control sample showing a vasicentric parenchyma cell with a nucleus, chromatin-like body, mitochondria, endoplasmic reticulum, and starch grains. Note apparent striations in secondary cell walls and protective layer. Line scale represents 2 microns.

Figure 8. *U. americana*, from control sample showing ray parenchyma cell. Note the nondiscolored pit "membrane". Line scale represents 1 micron.
Figure 9. U. americana, from control sample showing abundant starch grains in parenchyma cells. Line scale represents 1 micron.
Figure 10. *U. carpinifolia* 'Christine Buisman', from control sample showing normal cellular organelles. This photomicrograph is the same as Figure 12 but showing more of the cell wall with faint striations. Line scales represents 1 micron.

Figure 11. *U. carpinifolia* 'Christine Buisman', from control sample showing normal cellular organelles. Line scale represents 1 micron.
Figure 12. *U. carpinifolia* 'Christine Buisman', from control sample. Note nucleus, mitochondria, and the large starch grains within the parenchyma cell. Line scale represents 1 micron.
Figure 13. *U. americana*, 11 days after inoculation showing tyloses within vessels. Note the gum-like material within the vessel and deterioration of parenchyma cells. Line scale represents 5 microns.

Figure 14. *U. americana*, 11 days after inoculation showing deterioration of ray parenchyma cells and tyloses. Line scale represents 5 microns.

Figure 15. *U. americana*, 11 days after inoculation showing a tyloses within a vessel. Note the secondary cell wall and discoloration within a tylosis. Line scale represents 5 microns.
Figure 16. *U. americana*, 14 days after inoculation. Note the several tyloses within a single vessel and the deterioration of tyloses and parenchyma. Line scale represents 5 microns.

Figure 17. *U. carpinifolia 'Christine Buisman'*, 14 days after inoculation. Note discoloration and thickening of vessel walls (small arrows) and deterioration of ray parenchyma cells. Line scale represents 5 microns.
Figure 18. *U. carpinifolia* 'Christine Buisman', 17 days after inoculation. Note the discoloration of ray parenchyma cells. Line scale represents 5 microns.

Figure 19. *U. carpinifolia* 'Christine Buisman', 20 days after inoculation. Note discoloration of vessel wall surfaces and intertracheary pit "membranes". Line scale represents 5 microns.
Figure 20. *U. americana*, 14 days after inoculation showing a vessel containing gum and a tylosis. Note parenchyma cells containing apparent starch grains. Line scale represents 5 microns

Figure 21. *U. americana*, 20 days after inoculation. Note discoloration of ray and vasicentric parenchyma cells. Line scale represents 5 microns
Figure 22. *U. americana*, 40 days after inoculation. Note thickened vessel walls, tissue deterioration, gums and discoloration in vessels, and parenchyma cells. Line scale represents 5 microns.

Figure 23. *U. americana*, 43 days after inoculation. Note the pit between the two tyloses located in the vessel. Line scale represents 5 microns.
Figure 24. *U. carpinifolia* 'Christine Buisman', 46 days after inoculation. Note gum-like material (small arrows) along vessel walls, and discoloration of vessel walls and pit "membranes". Line scale represents 5 microns.

Figure 25. *U. carpinifolia* 'Christine Buisman', 76 days after inoculation. Note abundant accumulation of phenolic-like material in parenchyma cells, and the formation of tyloses and gum-like material in vessels (small arrows). Line scale represents 5 microns.
Figure 26. *U. americana*, 43 days after inoculation showing the beginning of deterioration of the plasma-membrane. Note the apparent formation of vacuoles in the lower part of the cell and discoloration along the walls of the adjacent parenchyma cells. Line scale represents 1 micron.
Figure 27. *U. americana*, 43 days after inoculation. Note the distorted chloroplast-like structure, disruption of the nuclear membrane and the highly vacuolated parenchyma cell. Line scale represents 1 micron.
Figure 28. *U. americana*, 43 days after inoculation showing phenolic-like substance within a vessel and a parenchyma cell. Note the deterioration of the plasmamembrane and formation of vesicles in the chloroplast-like structure. Line scale represents 1 micron.
Figure 29. *U. americana*, 43 days after inoculation showing the formation of vesicles from the membrane systems of parenchyma cells. Note the separation of the nuclear membrane. Line scale represents 1 micron.
Figure 30. *U. americana*, 43 days after inoculation showing a parenchyma cell with vesicles and vacuoles. Note the distorted mitochondria within the tylosis. Line scale represents 1 micron.
Figure 31. *U. americana*, 43 days after inoculation showing a parenchyma cell with a highly discolored, deteriorating nucleus and discoloration throughout the cell lumen. Note the second-second cell wall of the parenchyma cell, zonations in the secondary cell wall, and possible deterioration of middle lamella. Line scale represents 1 micron.
Figure 32. *U. carpinifolia* 'Christine Buisman', 14 days after inoculation showing a parenchyma cell adjacent to the cambium. Note the formation of vesicles near the dictyosomes and throughout the cell, and apparent deterioration of cytoplasm. Line scale represents 1 micron.
Figure 33. Light microscopic cross-section of area associated with the section in Figure 34. Line scale represents 5 microns

Figure 34. *U. americana*, 40 days after inoculation showing starch grains in deteriorating and dead parenchyma cells. Line scale represents 1 micron
Figure 35. U. americana, 40 days after inoculation showing deterioration of the plasmamembrane to form vesicle-like structures. Note distorted chloroplast and tannin-like bodies within parenchyma cells. Line scale represents 1 micron.
Figure 36. *U. americana*, 43 days after inoculation showing tannin accumulation and starch grains in deteriorating and dead parenchyma cells. Note second-secondary cell walls in parenchyma cells. Line scale represents 1 micron.
Figure 37. *U. americana*, 43 days after inoculation showing tannin-like material in a parenchyma cell. Note distorted chloroplast and mitochondria, and formation of vacuoles. Line scale represents 1 micron.
Figure 38. *U. americana*, 43 days after inoculation showing tannin-like bodies in parenchyma cells. Line scale represents 1 micron.
Figure 39. *U. carpinifolia* 'Christine Buisman', 17 days after inoculation showing rapid deterioration of the parenchyma cell. Note the presence of nucleus, mitochondria, dictyosome and starch grains. Line scale represents 1 micron.
Figure 40. *U. carpinifolia* 'Christine Buisman', 17 days after inoculation. Note the discoloration found throughout the parenchyma cell, deteriorating nucleus and starch grains. Line scale represents 1 micron.
Figure 41. *U. carpinifolia 'Christine Buisman*', 17 days after inoculation showing abundant phenolic accumulation within parenchyma cells. Line scale represents 1 micron.
Figure 42. *U. americana*, 43 days after inoculation showing vacuoles that contain phenolic-like substances. Note the formation of vesicles from the plasmamembrane located in the parenchyma cell and tylosis in adjacent vessel; and discoloration of intertracheary pit "membranes" and vessel wall surface. Line scale represents 1 micron.
Figure 43. Light microscopic cross-section of area associated with the section in Figure 44. Line scale represents 5 microns.

Figure 44. *U. americana*, 43 days after inoculation showing 2 tyloses in a vessel. Note the tylosis containing phenol-like material in vacuoles, and also starch grains. Line scale represents 1 micron.
Figure 45. Light microscopic cross-section of area associated with section in Figure 46. Line scale represents 5 microns

Figure 46. U. americana, 43 days after inoculation showing a tylosis within a vessel. Note the numerous vesicle-like structures, distorted mitochondria (small arrows) and large vacuole within expanded tylosis. Line scale represents 1 micron
Figure 47. *U. americana*, 43 days after inoculation showing discolored parenchyma cells adjacent to a vessel containing a highly vacuolated tylosis. Line scale represents 1 micron.
Figure 48. Light microscopic cross-section of area associated with section in Figure 49. Line scale represents 5 microns.

Figure 49. *U. carpinifolia 'Christine Buisman',* 46 days after inoculation showing the diffusion of phenol-like or gum-like material into a vessel from an adjacent parenchyma cell. Note the vacuoles and distorted mitochondria. Line scale represents 1 micron.
Figure 50. *U. carpinifolia* 'Christine Buisman', 17 days after inoculation showing a deteriorating parenchyma cell and a vessel containing gum-like material between cell wall and a tylose. Note the deterioration of the plasmamembrane and nuclear membrane. Line scale represents 1 micron.
Figure 51. *U. americana*, 11 days after inoculation showing gum-like material against vessel wall. Line scale represents 1 micron.
Figure 52. Light microscopic cross-section of a vessel, as seen in Figure 53. Line scale represents 5 microns.

Figure 53. *U. americana*, 20 days after inoculation showing granular, gum-like material or deteriorated fungus appressed against the vessel wall by a tylosis. Line scale represents 1 micron.
Figure 54. *U. americana*, 20 days after inoculation showing the accumulation of gum-like material or deteriorated fungus along the vessel wall. Line scale represents 1 micron.
Figure 55. *U. carpinifolia 'Christine Buisman'* , 76 days after inoculation showing gum-like material on vessel walls and phenol-like or gum-like material throughout the lumen. Note the discolored intertracheary pit "membranes". Line scale represents 1 micron.
Figure 56. *U. carpinifolia 'Christine Buisman*', 76 days after inoculation showing discoloration of cytoplasm and formation of vacuoles within a parenchyma cell. Note the layers in the secondary cell wall and the formation of a second-secondary cell wall. Line scale represents 1 micron.
Figure 57. *U. americana*, 40 days after inoculation showing the deterioration of parenchyma cells, second-secondary cell wall of the parenchyma cell, lack of a secondary cell wall in the parenchyma cell adjacent to the vessel with tylosis, and possible cross-section of microhyphae in cell walls. Line scale represents 2 microns.
Figure 58. U. americana, 40 days after inoculation showing discoloration of vessel walls and intertracheary pit "membranes", and possible microhyphae in wall. Line scale represents 2 microns.
Figure 59. *U. americana*, 43 days after inoculation. Note the layers of the secondary cell walls and the "tearing" in the middle lamella area of the cell wall, and possible microhypha in wall. Line scale represents 1 micron
Figure 60. *U. americana*, 43 days after inoculation. This photomicrograph represents an enlargement of Figure 59 showing "tearing" in the middle lamella area of wall. Line scale represents 1 micron.
Figure 61. *U. americana*, 43 days after inoculation. This photomicrograph represents an enlargement of Figure 59 showing possible microhypha in wall. Line scale represents 1 micron.
Figure 62. *U. americana*, 43 days after inoculation showing an apparent gradation from a deteriorating parenchyma cell to one containing tannin. Note the deterioration of the plasmamembrane and nuclear membrane. Line scale represents 1 micron.
Figure 63. *U. americana*, 43 days after inoculation. Note the gradation from an apparent normal parenchyma cell to a dead one containing only tannin; and the formation of a second-secondary cell wall and layers in cell walls. Line scale represents 1 micron.